(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 April 2001 (19.04.2001)

PCT

(10) International Publication Number
WO 01/27321 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/NL00/00737

(22) International Filing Date: 13 October 2000 (13.10.2000)

(25) Filing Language: Dutch

(26) Publication Language: English

(30) Priority Data:
1013286 13 October 1999 (13.10.1999) NL

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/27321 A2

(54) Title: METHOD FOR DETECTING DIFFERENCES IN GENE EXPRESSION BETWEEN WILD TYPE PLANTS AND NEWLY DEVELOPED PLANTS

(57) Abstract: The present invention relates to a method for detecting differences in gene expression between wild type plants and newly developed plants, comprising of: a) providing at least one micro-array which is provided with a number of spots of target material; b) proividing at least one probe which comprises test material of a wild type plant, and a probe which comprises test material of a newly developed plant; c) placing on the micro-array the target material spotted thereon in contact with the probes; d) determining per spot whether hybridization of the probe of the wild type plant with the target material has taken place and determining per spot whether hybridization of the probe of the newly developed plant with the target material has taken place, in order to determine whether there is a difference in gene expression between the wild type plant and the newly developed plant, characterized in that the test material comes from plants which have been subjected to a treatment prior to isolation of the test material. With the method according to the invention it is possible to demonstrate in simple manner to what extent the gene expression of a newly developed plant differs, as a consequence of a worst case treatment, from the corresponding wild type plant.

METHOD FOR DETECTING DIFFERENCES IN GENE EXPRESSION BETWEEN WILDTYPE PLANTS AND NEWLY DEVELOPED PLANTS

The present invention relates to a method for detecting differences in gene expression between wild type plants and newly developed plants.

Plants generally adapt to changes in their environment by a change in expression of various genes and the production of various proteins. Changes in environmental conditions can for instance result in expression of genes which are first not expressed, or only to a small extent (up-regulation), or in suppression of the expression of certain genes (down-regulation). In newly developed, such as for instance genetically modified plants, certain genes can however be lost due to the introduction of a new gene, whereby the newly developed plant can respond in a different way to environmental conditions. It is known to study environmental effects on newly developed plants (mutants, genetically modified plants, or improved plants obtained via classical improvement) by studying the pleiotropic environmental effects on the gene expression of the relevant plants after incubation of the plants under differing climate-dependent environmental conditions. These effects are studied at the level of DNA-expression, for instance using DNA micro-arrays, and/or at the level of the plant as a whole through evaluation of the phenotype or analysis of the metabolic composition. This approach is however insufficient to determine the full potential of pleiotropic effects on the gene expression of the newly developed plants because the simultaneous prevention of a number of environmental conditions which can result in expression of the full potential of harmful (adverse) effects depends on chance (e.g. wounding, possible infections with pathogenic micro-organisms, air pollution, flood, drought, frost, nutrient deficiency or excess etc.). Testing at different locations during two or more growing seasons does not always result in

conditions in which the full potential of possible (harmful) pleiotropic effects is expressed.

The object of the present invention is to obviate the above stated drawback.

This objective is achieved with the invention by providing a method for detecting differences in gene expression between wild type plants and newly developed plants, comprising the steps of:

a) providing at least one micro-array which is provided with a number of spots of target material;

b) providing at least one probe which comprises test material of a wild type plant, and a probe which comprises test material of a newly developed plant;

c) placing on the micro-array the target material spotted thereon in contact with the probes;

d) determining per spot whether hybridization of the probe of the wild type plant with the target material has taken place and determining per spot whether hybridization of the probe of the newly developed plant with the target material has taken place, in order to determine whether there is a difference in gene expression between the wild type plant and the newly developed plant, wherein the test material comes from plants which have been subjected to a treatment prior to isolation of the test material. With the method according to the invention it is possible to demonstrate in a simple manner to what extent the gene expression of a newly developed plant differs, as a consequence of the treatment, from the corresponding wild type plant.

Micro-arrays generally consist of large numbers (thousands) of DNA, cDNA or RNA fragments arranged on a glass plate as small "spots", designated as target material. The sequence of the fragments and the function of the associated genes can be analyzed. Using the micro-array it is possible to demonstrate via standard DNA-hybridization techniques which genes are active in a particular tissue under certain conditions. For this purpose the target material is hybridized with DNA, cDNA, or RNA-probes comprising the tissue for testing provided with a label (the test material). Due to the

label it is readily possible to determine whether hybridization of the test material with the target material has taken place. The intensity of the coloring is herein proportional to the (relative) expression level of the gene. A fluorescent label can for instance be used as label.

In the method according to the invention a probe, which comprises the test material from an existing plant, the wild type plant, and a probe, which comprises the test material from a newly developed plant, are tested simultaneously on a micro-array. The probe of the wild type plant is for instance labeled for this purpose with a yellow fluorescent label and the test material of the newly developed plant with a red fluorescent label. Depending on the color of the different spots of the target material after hybridization, an assessment can be made as to the expression of determined genes in the different plants, and in particular as to the differences in the gene expression between the wild type plant and the newly developed plant. Spots which are for instance yellow or red on the micro-array after the hybridization indicate that the relevant target material is expressed in only one of the two plants. The spots with an intermediate (orange) color indicate that the target material from this spot is expressed in both the wild type plant and the newly developed plant. Using optical equipment the color can be determined very accurately and the relative difference in expression between the plants can be determined per spot. The micro-array technique enables simultaneous detection of thousands of genes and analysis of the expression thereof.

The method according to the invention can for instance be used to detect differences in gene expression between wild type and newly developed plants in respect of certain improvement properties, for ecological risk-analyses, to determine the genetic diversity of plant populations, or to determine the (nutritional) safety of particular newly developed crops. The method according to the invention is of

particular importance in determining the ecological risks, the nutritional safety and toxicity of genetically modified plants (GMPs).

The treatment to which the plants are subjected prior to the isolation of the test material is preferably a stress-inducing treatment. Pleiotropic effects on for instance the development, growth, crop yield, susceptibility to diseases, nutritional safety and ecological risk etc. generally become clearly manifest here. The plants are incubated under differing "worst case" conditions. These "worst case" conditions include incubation conditions wherein the plants are exposed to an optimal stress, whereby the plants are forced to express all genes related to these pleiotropic effects. The maximum potential of pleiotropic effects of a changed environment on the gene expression of the plants can thus be established.

The genes identified with the method according to the invention can be used for instance as markers for the relevant pleiotropic effects. These markers can then for instance be used by plants breeders to enable a rapid and reliable selection of elite GMPs, mutants or otherwise improved plants. The number of field experiments can hereby be reduced.

It is moreover possible with the method according to the invention to assess the potential of the newly developed plants to run wild, i.e. the (undesirable) capacity of the newly developed plants to spread.

The method according to the invention is of great importance for determining the nutritional safety of newly developed plants for humans and animals. The effect of an introduced gene on the expression of stress and toxicity-related genes can be measured quantitatively. It can thus be determined for instance whether, under "worst case" conditions, the types and levels of anti-nutritional components and natural toxins ("anti-feedants") differ in genetically modified plants from those of the associated "wild type" plants.

The method according to the invention can for instance also be further used to screen ecosystems under

differing physiological and ecological conditions, or to determine the genetic diversity among plant populations. Ecosystems with little genetic diversity are vulnerable under worst case conditions. Depending on the information obtained using the method according to the invention, these ecosystems can for instance be improved by developing specific planting programs.

The target material and the test material can comprise DNA, cDNA and RNA. The suitable test material can be chosen subject to the target material used.

In a particular embodiment of the invention the target material comprises protein, and the test material at least one antibody specific for the protein. In this manner it is possible to test whether there are differences at protein level between the wild type plants and the newly developed plants.

As described above, use can be made in the method according to the invention of for instance DNA and/or protein micro-arrays (DNA and/or protein "chips"). The DNA chip can for instance be made with cDNA which comes from mRNA of a wild type plant after a stress-inducing treatment and DNA molecules, or parts of DNA molecules, which are related to the genes for study (e.g. genes involved in the forming of toxins or anti-nutrients). ESTs ("expressed sequence tags") which are related to the genes for study can also be used. Many ESTs are available commercially. Usable arrays can also be obtained commercially.

Before the test material is isolated from the plants to be studied, the plants are subjected to a stress-inducing treatment. It is important here that the wild type plants and the newly developed plants are treated in practically identical manner. Use can for instance be made for this purpose of a randomized experimental set-up in climate chambers.

After the treatment of the plants, the gene expression is analyzed of both the wild type plants, from which the material of the chip originates, and the newly developed plants (mutants, genetically modified (transgenic) plants or plants obtained via classical

breeding methods). The differences in gene expression, after the different treatments, between the wild type plant and the newly developed plant demonstrate to what extent the gene expression is influenced by the genetic transformation. Genes (or clusters of genes) which are partially expressed can then be sequenced and identified and can for instance be used as markers. Additional research can if necessary be carried out, depending on the function of the gene, such as specific toxicological, ecological or physiological research.

Plants can be induced to express their full potential of genes involved in the differing pleiotropic effects of environmental conditions, such as for instance genes which are involved in the production of toxins, by cultivating them under special conditions. In a preferred embodiment of the method according to the invention the stress-inducing treatment comprises the exposure of the plants to stress-inducing compounds. The induction of the biosynthesis of undesirable substances or processes can be effected with determined stress-inducing compounds such as "signal molecules" which are involved in the signal-transduction of stress. Examples of suitable stress-inducing compounds are, among others, jasmonic acid, salicylic acid, ethylene, elicitors (compounds which stimulate the plant immune system) and oxidative stress inducers, such as for instance paraquat or UV-light. Combinations of stress-inducing compounds can also be used.

In another preferred embodiment of the invention the stress-inducing treatment comprises the exposure of the plants to maximum agronomic stress. Agronomic stress relates to the stress which can occur in agriculture and horticulture, for instance due to unforeseen circumstances. Agronomic stress can for instance be induced by cultivating the plants under particular combinations of nutrition (for instance nitrogen), light, temperature, water, drought, pathogenic micro-organisms etc).

The method according to the invention enables simultaneous analysis of the expression of thousands of

genes which are expressed before and after a stress-inducing treatment, and a very precise assessment can therefore be made in respect of risk-analysis and the safety of genetically modified plants. This can also be done for other possible undesired effects, such as concealed improvement effects.

The term "newly developed plant" according to the present invention relates to any new plant whose genetic material differs in any way from that of the corresponding wild type plant. Newly developed plants according to the invention can for instance be mutant plants (spontaneous or induced), or plants modified by means of recombinant DNA-techniques, but can for instance also be newly developed plants obtained via classical improvement techniques.

For the method according to the invention the test material can be isolated from all parts of newly developed plants, such as for instance the root, stem, leaf, flower, pollen, fruits, seeds etc.

A good stress response plays an important part in the ability of the plant to survive in adverse environmental situations. In stress situations the plant will generally produce toxic "defence molecules" (so-called phytoalexins). In the toxicological evaluation of newly developed plants it is necessary to ensure that sufficient genes and/or proteins related to the production of such toxic substances (such as inter alia alkaloids, particular peptides, lectins, proteinase inhibitors) are present on the array. If after the treatment differences in expression levels occur between the wild type plants and the newly developed plants, an additional specific toxicological test can optionally be carried out.

In order to determine the effects of stress on the plants, it is of further importance that the genes and/or proteins related to the production of stress-substances (such as inter alia flavonoids, anthocyanins, salicylic acid, jasmonate, elicitors, ethylene, secondary metabolites) are present on the micro-array. On the basis of differences in expression levels between

wild type and newly developed plants after the stress-inducing treatment it is possible to assess the chance of running wild, or the chance of for instance the production of anti-nutrients, and if desired additional ecological, plant-pathological and/or toxicological research can be carried out.

For the method according to the invention diverse different stress-inducing "worst case" treatments can be applied and the effect thereof on the gene expression of the newly developed plants can be tested.

If great differences can be seen on the micro-array, or a large number of genes are changed, the method according to the invention can be further combined with additional research into changes in important physiological processes of the plants, such as for instance photosynthesis, apical domination, form of the plant, plant-plant interaction, regeneration capacity and seed production.

The invention is further elucidated on the basis of the following example.

EXAMPLE

In this example the method according to the invention was applied to the tomato cultivar Ailsa Craig (the wild type; "wt") and two mutants of this cultivar: the "green flesh" ("GF") and the "high pigment" ("HP") mutant. These mutants are well described in the literature and it is known which genes are changed (Terry and Kendrick 1999, Plant Physiology 119: pp. 143-152; Peters et al. 1998, Plant Physiology 117: pp. 797-807; Lazarova et al. 1998, Plant Molecular Biology 38: pp. 1137-1146; Lazarova et al. 1998, The Plant Journal 1998,14: 653-662; Yang et al. 1998, Planta 206: 685-688).

The phenotypical effect of the mutations under normal (standard) growth conditions was examined at the moment the plants were fully grown and carried the ripe fruits (table 1). The GF mutant strongly resembled the wt Ailsa Craig. The flesh of this mutant was however

slightly greener (appeared less ripened). The GF plants were also somewhat lighter in weight and shorter than the wild type Ailsa Craig plants.

The HP mutant differed phenotypically more from the wild type than the GF mutant (table 1). The HP plants were clearly lighter in weight and shorter than the standard plants and had more brown/red pigment in stem and leaves. The leaves moreover contained more chlorophyl, as measured with the spat-meter.

The three types of Ailsa Craig plants (wt, GF and HP) were incubated in a climate chamber both under normal conditions and under extreme agronomic stress conditions (randomized set-up, n=6). The agronomic stress was induced 8 weeks after the tomato seeds were germinated. During this first 8 weeks all plants were incubated under optimal conditions.

For the whole culture period the plants were incubated from 08.00 to 22.00 hours at 20°C, and from 22.00 to 08.00 hours ("night" period) at 16°C at an air humidity of 70%. The plants were in pots (diameter 20 cm) with potting compost on dishes with Steiner nutrient solution (for composition see: proceedings of the sixth international congress on soil-less culture, Lunteren 1984, published by the secretariat of ISOSC, P.O. Box 52, 6700 AB Wageningen, The Netherlands, pp. 633-649). For the first 8 weeks care was taken that the dishes did not dry out.

A first group of plants (wt, HP and GF; n=6) was further cultivated under these conditions until ripe tomatoes could be harvested. A second group of plants (wt, HP and GF; n=6) was exposed to maximum agronomic stress in respect of water and nutrients. This was achieved by reducing the amount of nutrient solution applied, as shown in table 2.

Ripe tomatoes were harvested six weeks after the start of the stress-induction. Tomatoes coming from three plants of one particular type were pooled. Each tomato was cut into 8 pieces, frozen quickly in liquid nitrogen and optionally stored at -80°C.

mRNA from the three groups of plants (wt, GF, HP) was extracted using Dyna-beads (Dynal) after incubation under stress as well as after incubation without agronomic stress. This mRNA was used for the μ-array analyses. In each array mRNA from Ailsa Craig wt without stress was used as control. The mRNA labeling, μ-array incubation and analysis were performed as described by Schena et al. 1995, Science (Washington DC) 270(5235): pp. 467-470. The μ-array was made from genes which are expressed in ripe tomato fruits.

Results:

Effect of the stress treatment on plant development

The stress treatment resulted for each group of plants (wt, GF and HP) in a very large reduction in fresh weight (± 75%). The length (± 50%) and the number of leaves (±25%) also decreased considerably (table 3). The plants cultivated under stress had more chlorophyl on average (± 10 to 20%) than the plants without stress. The different plant types reacted in similar manner to the stress treatment. However, the flesh of the GF plants after stress was clearly less green than that of plants cultivated without stress and in terms of color resembled the flesh of control tomatoes. The HP plants cultivated under stress could be immediately distinguished with the naked eye from the control plants, because they were a much darker purple/red/brown in color; they were moreover also shorter than the GF plants which were incubated with the stress-inducing treatment.

Analysis of gene expression on the μ-array

A total of 200 fragments of different genes and extra control DNA were spotted on a microscopic slide. In this analysis the effect of the induced stress on the gene expression of GF and HP plants was compared with the effect thereof on wt plants. It is thus possible to check whether there are, and if so what effects the

mutations have on the expression of genes present on the array.

Effect of agronomic stress on down-regulation of gene expression of GF and HP plants compared to wt plants

Under the influence of stress, genes were down-regulated in both the HP plants and GF plants: in the case of the HP plants one gene, and in the case of the GF plants three genes. The limit for down-regulation was at an average expression value which was more than 50% lower than the expression value in wt plants incubated under stress. One gene was down-regulated in both mutants: the gene which codes for lipoic acid synthase (Arabidopsis). Lipoic acid synthase is a coenzyme which is essential for the activity of enzyme complexes, such as of pyruvate dehydrogenase and glycine decarboxylase. Pyruvate dehydrogenase plays a part in the cell-respiration and glycine decarboxylase is a photo-respirating protein which is expressed in green plant tissue. Down-regulation of these enzymes can indicate accelerated (further) ripening of the fruit in the GF and HP tomatoes compared to the wt tomatoes.

Without stress conditions ten genes were down-regulated in the HP plants and one gene in the GF plants. This can indicate that the HP tomatoes were further ripened than the wt tomatoes. In ripened fruits fewer enzymes are necessary to maintain the many metabolic processes which occur to a greater extent in young fruits.

Effect of agronomic stress on up-regulation of gene expression of GF and HP plants compared to wt plants

Under stress 16 genes were up-regulated in the case of the HP plants and 4 in the case of the GF plants, compared with the wt stress control plants. Taken as limit was an increase in gene expression of more than 50% compared to the control plants. Under the influence of stress the phenotype of HP mutant changed considerably when compared with the changes in the wt and GF plants: the plants had a different colour and

were shorter and less heavy (see table 3). The HP mutation evidently also results in the case of stress in much interaction with genes which are expressed in the fruit.

4-hydroxyphenylpyruvate dioxygenase is up-regulated in the HP mutant. This enzyme is involved in the carotenoid biosynthesis and possibly contributes toward the typical colour of the HP plants under stress.

Without stress 2 genes were up-regulated in the HP plants and 11 in the GF plants compared with the wt plants. This large number of genes which is up-regulated without stress in the GF compared with the wt plants can be explained in that the GF tomato fruit tissue is green and that of the wt plants is not. In the GF flesh there are evidently situated many cells which are physiologically young and in which diverse enzyme systems are still active. In the GF plants which were incubated under the influence of stress the flesh is no longer green (table 3). The fruits of GF plants which were incubated under stress thus more closely resembled wt fruits than GF fruits incubated without stress. A relatively large number of pleiotropic effects were found at gene expression level (particularly in the case of the HP mutant).

With the method according to the invention differences in gene expression, resulting from diverse "worst case" conditions, between plants which differ from each other in only one gene (mutant or GMO) or a plurality of genes (cross-breeding products) can thus be studied in simple manner. A detailed analysis of the gene function of the up or down-regulated genes can provide the answer to the question of which processes differ, and the extent to which these processes differ, between the newly developed plant and the existing wildtype plant.

Table 1. Effect of the HP and GF mutation on the development of the tomato (cv Ailsa Craig).

Property	Control plant "Wt "	HP mutant	GF mutant
weight (gram±SD)	1653±233	1284±268	1539±316
chlorophyl upper leaves (arbitrary units spatmeter)	49.1±5.5	55±3.5	48.8±3.5
chlorophyl middle leaves (arbitrary units spatmeter)	51.3±4.6	57.7±2.9	51.9±4.3
amount of leaves per plant	29.6±2.0	25.4±4.3	26.2±2.0
length (cm)	194.6±8.8	150.2±31.3	170.5±13.6
color plant	green	a little darker brown/purple	green like control
color flesh	normal flesh color	normal flesh color	flesh clearly green

Table 2. Amount of Steiner nutrient solution during agronomic stress.

Time (days) after start stress treatment	volume (cc) nutrient solution with stress induction	volume nutrient solution without stress induction
1	240	1500
2	250	1500
3	250	0
4	250	1000
5	250	1000
6	250	1000
7	250	1000
8	0	1000
9	250	0
10	250	1000
11	250	1000
12	125	0
13	125	1000
14	125	1000
15	125	1000
16	125	500
17	125	1000
18	125	1000
19	125	1000
20	125	1500
21	125	0
22	125	1000
23	200	1500
24	200	1500
25	200	1000
26	200	0

27	250	1000
28	250	1000
29	200	1500
30	0	0
31	200	1500
32	200	1500
33	200	1500
34	200	1500
35	200	1500
36	200	1500
37	200	1500
38	200	1500
39	200	1500
40	200	1500
41	200	1500
42	0	0
43	200	1500
44	200	1500

Table 3. Effect of the HP and GF mutation on plant development in tomato (cv Ailsa Craig) after incubation under optimal conditions and under conditions of maximal agronomic stress. (All values are averages of 6 assays ± SD).

	without stress			with stress		
property	control plant "wildtype"	HP mutant	GF mutant	control plant "wildtype"	HP mutant	GF mutant
weight (gram)	1653±233	1284±268	1539±316	334±23	337±26	349±27
chlorophyl upper leaves (arbitrary units spatmeter)	49.1±5.5	55±3.5	48.8±3.5	61.2±3.6	62.6±6.6	62.4±3.7
chlorophyl middle leaves (arbitrary units spatmeter)	51.3±4.6	57.7±2.9	51.9±4.3	56.8±3.7	61.2±3.6	58.1±3.9
amount of leaves per plant	29.6±2.0	25.4±4.3	26.2±2.0	17.4±2.4	17.0±1.6	16.4±1.9
length (cm)	194.6±8.8	150.2±31.3	170.5±13.6	126.7±26.8	94.3±16.7	111.3±8.0
color plant	green	a little darker brown/ purple	green control	green		green
color flesh	normal color flesh	normal color flesh	clearly green	normal color flesh	normal color flesh	normal color flesh

CLAIMS

1. Method for detecting differences in gene expression between wild type plants and newly developed plants, comprising the steps of:

a) providing at least one micro-array which is provided with a number of spots of target material;

b) providing at least one probe which comprises test material of a wild type plant, and a probe which comprises test material of a newly developed plant;

c) placing on the micro-array the target material spotted thereon in contact with the probes;

d) determining per spot whether hybridization of the probe of the wild type plant with the target material has taken place and determining per spot whether hybridization of the probe of the newly developed plant with the target material has taken place, in order to determine whether there is a difference in gene expression between the wild type plant and the newly developed plant, **characterized in that** the test material comes from plants which have been subjected to a treatment prior to isolation of the test material.

2. Method as claimed in claim 1, **characterized in that** the treatment is a stress-inducing treatment.

3. Method as claimed in claim 2, **characterized in that** the stress-inducing treatment is a worst-case treatment.

4. Method as claimed in claim 2 or 3, **characterized in that**, the stress-inducing treatment comprises the exposure of the plants to one or more stress-inducing compounds.

5. Method as claimed in claim 4, **characterized in that**, the stress-inducing compound comprises jasmonic acid, salicylic acid, ethylene, an elicitor and/or an oxidative stress inducer.

6. Method as claimed in claim 3, **characterized in that** the stress-inducing treatment comprises the exposure of the plants to agronomic stress.

7. Method as claimed in claims 1-6, **characterized in that** the target material comprises DNA, cDNA, or RNA.

8. Method as claimed in claims 1-6, **characterized in that** the test material comprises DNA, cDNA, or RNA.

9. Method as claimed in claims 1-6, **characterized in that** the target material comprises protein, and the test material comprises at least one antibody specific for the protein.

10. Method as claimed in claims 1-9, **characterized in that** the newly developed plant is a mutated plant, a genetically modified plant or a new plant obtained by means of classical improvement.

11. Method as claimed in claims 1-10 for determining the nutritional safety of newly developed plants for humans and animals.

12. Method as claimed in claims 1-10 for determining the ecological risks of newly developed plants.

13. Method as claimed in claims 1-10 for determining concealed improvement properties of newly developed plants.